09-13-00 A/Box Seq

DOCKET NO.: RTS-0201

"Express Mail" Label No.: EL263653945US
Date of Deposit: 9/11/2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Hong Zhang and Andrew T. Watt

For: **Antisense Modulation of caspase 7 Expression**

BOX SEQUENCE
Assistant Commissioner for Patents
Washington DC 20231

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find the following:

- The specification of the above-referenced patent application;
- An **executed** Declaration or Oath and Power of Attorney;
- An Assignment of the invention to **Isis Pharmaceuticals Inc.** with recordation cover sheet (PTO Form PTO-1595) and $40.00 cover fee;
- An **executed** Verified Statement Claiming Small Entity Status under 37 CFR 1.9 and 1.27;
- Statement to Support Filing and Submission of DNA/Amino Acid Sequences in Accordance with 37 CFR § § 1.821 through 1.825;
- Sequence listing in computer readable form in accordance with 37 C.F.R. § 1.821(e); and
- An Information Disclosure Statement with references.

The filing fee has been calculated as shown below:

For:	No. Filed	No. Extra	Rate	Fee
BASE FEE				$345.00
Total Claims	20 - 20 =	0	X $9=	$0
Indep. Claims	2 - 3 =	0	x$39=	$0
			TOTAL	$ 345.00

The Commissioner is hereby authorized to charge the following fees to Deposit Account No. 500252:

- the amount of $385.00 for the above listed fees;
- payment of the following fees associated with this communication or credit any overpayment;
- any additional filing fees required under 37 CFR 1.16 including fees for presentation of extra claims; and
- any additional patent application processing fees under 37 CFR 1.17 and under 37 CFR 1.20 (d).

Triplicate copies of this transmittal are enclosed.

Date: Sept. 11 2000

Laurel Spear Bernstein
Registration No. 37,280
Isis Pharmaceuticals, Inc.

Please address all correspondence to:
Jane Massey Licata
Law Offices of Jane Massey Licata
66 East Main Street
Marlton NJ 08053
(856) 810-1515

DOCKET NO.: RTS-0201

"Express Mail" Label No.: EL263653945US
Date of Deposit: 9/11/2000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Hong Zhang and Andrew T. Watt

For: **Antisense Modulation of caspase 7 Expression**

BOX SEQUENCE
Assistant Commissioner for Patents
Washington DC 20231

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find the following:

- The specification of the above-referenced patent application;
- An **executed** Declaration or Oath and Power of Attorney;
- An Assignment of the invention to **Isis Pharmaceuticals Inc.** with recordation cover sheet (PTO-Form PTO-1595) and $40.00 cover fee;
- An **executed** Verified Statement Claiming Small Entity Status under 37 CFR 1.9 and 1.27;
- Statement to Support Filing and Submission of DNA/Amino Acid Sequences in Accordance with 37 CFR § § 1.821 through 1.825;
- Sequence listing in computer readable form in accordance with 37 C.F.R. § 1.821(e); and
- An Information Disclosure Statement with references.

The filing fee has been calculated as shown below:

For:	No. Filed	No. Extra	Rate	Fee
BASE FEE				$345.00
Total Claims	20 - 20 =	0	X $9=	$0
Indep. Claims	2 - 3 =	0	x$39=	$0
			TOTAL	$ 345.00

The Commissioner is hereby authorized to charge the following fees to Deposit Account No. 500252:

- the amount of $385.00 for the above listed fees;
- payment of the following fees associated with this communication or credit any overpayment;
- any additional filing fees required under 37 CFR 1.16 including fees for presentation of extra claims; and
- any additional patent application processing fees under 37 CFR 1.17 and under 37 CFR 1.20 (d).

Triplicate copies of this transmittal are enclosed.

Date: Sept. 11 2000

Laurel Spear Bernstein
Registration No. 37,280
Isis Pharmaceuticals, Inc.

Please address all correspondence to:
Jane Massey Licata
Law Offices of Jane Massey Licata
66 East Main Street
Marlton NJ 08053
(856) 810-1515

DOCKET NO.:RTS-0201

"Express Mail" Label No.: EL263653945US
Date of Deposit: 9/11/2000

Applicant or Patentee: Hong Zhang and Andrew T. Watt

Serial or Patent No.: not yet assigned **Date Filed or Issued:** herewith

For: Antisense Modulation of caspase 7 Expression

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c) - SMALL BUSINESS CONCERN)

I hereby declare that I am an official empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Isis Pharmaceuticals Inc.
ADDRESS OF CONCERN: 2292 Faraday Ave
Carlsbad, California 92008

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that: (1) the number of employees of the concern, including those of its affiliates, does not exceed 500 persons; and (2) the concern has not assigned, granted, conveyed, or licensed, and is under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who could not be classified as an independent inventor if that person had made the invention, or to any concern which would not qualify as a small business concern or a nonprofit organization under this section.

For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled **Antisense Modulation of caspase 7 Expression** by inventor(s) Hong Zhang and Andrew T. Watt described in the specification filed herewith.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

***NOTE: Separate verified statements are required for each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)**

FULL NAME: **( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN**
ADDRESS: **( ) NONPROFIT ORGANIZATION**

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING C. Frank Bennett
TITLE OF PERSON SIGNING Vice President, Biology
ADDRESS OF PERSON SIGNING 2292 Faraday Avenue
Carlsbad, CA 92008

C. Frank Bennett
SIGNATURE

9-6-00
DATE

ANTISENSE MODULATION OF CASPASE 7 EXPRESSION

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of caspase 7. In particular, this invention relates to compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding caspase 7. Such compounds have been shown to modulate the expression of caspase 7.

BACKGROUND OF THE INVENTION

Apoptosis, or programmed cell death, is a naturally occurring process that has been strongly conserved during evolution to prevent uncontrolled cell proliferation. This form of cell suicide plays a crucial role in ensuring the development and maintenance of multicellular organisms by eliminating superfluous or unwanted cells. However, if this process goes awry becoming overstimulated, cell loss and degenerative disorders including neurological disorders such as Alzheimers, Parkinsons, ALS, retinitis pigmentosa and blood cell disorders can result. Stimuli which can trigger apoptosis include growth factors such as tumor necrosis factor (TNF), Fas and transforming growth factor beta (TGFβ), neurotransmitters, growth factor withdrawal, loss of extracellular matrix attachment and extreme fluctuations in intracellular calcium levels (Afford and Randhawa, *Mol. Pathol.*, **2000**, *53*, 55-63).

Alternatively, insufficient apoptosis, triggered by growth factors, extracellular matrix changes, CD40 ligand, viral gene products neutral amino acids, zinc, estrogen and androgens, can contribute to the development of cancer, autoimmune disorders and viral infections (Afford and Randhawa, *Mol. Pathol.*, **2000**, *53*, 55-63). Consequently, apoptosis is regulated under normal circumstances by the interaction of gene products that either induce or inhibit cell death and several gene products which modulate the apoptotic process have now been identified.

The most well-characterized apoptotic signaling cascade to date is that orchestrated by a family of cysteine proteases known as caspases. These enzymes activate apoptosis through proteolytic events triggered by one of several described mechanisms; including ligand binding to the cell surface death receptors of either the TNF or NGF receptor families, changes in mitochondrial integrity or chemical induction (Thornberry, *Br. Med. Bull.*, **1997**, *53*, 478-490).

Caspases have been classified into two groups, initiator caspases and effector caspases, based upon their position in the apoptotic signaling pathway.

Initiator caspases include caspase 1, 2, 4, 5, 8, 9, 10 and 14 and these enzymes have the largest prodomains of all the caspase zymogens. These prodomains allow the initiator caspases to interact with other downstream substrates including other caspases. Initiator caspases are further divided into two groups based on their protein binding domains. Caspases 8 and 10 contain the DED (death effector domain) while caspases 1, 2, 4 and 9 contain the CARD (caspase recruitment domain) (Bratton et al., *Exp. Cell. Res.*, **2000**, *256*, 27-33; Garcia-Calvo et al., *Cell. Death Differ.*, **1999**, *6*, 362-369).

Effector caspases are activated by initiator caspases and include caspase 3, 6, 7, 11 and 13 and these contain a shorter prodomain. Once activated, effector caspases then cleave a number of structural and regulatory proteins within

the cell (Bratton et al., *Exp. Cell. Res.*, **2000**, *256*, 27-33; Garcia-Calvo et al., *Cell. Death Differ.*, **1999**, *6*, 362-369).

Caspase 7 (also known as CASP7, MCH3, ICE-LAP3 and CMH-1) is an effector caspase expressed in all tissues except brain (Duan et al., *J. Biol. Chem.*, **1996**, *271*, 1621-1625; Fernandes-Alnemri et al., *Cancer Res.*, **1995**, *55*, 6045-6052; Juan et al., *Genomics*, **1997**, *40*, 86-93; Lippke et al., *J. Biol. Chem.*, **1996**, *271*, 1825-1828). Characterization of the gene revealed that caspase 7 exists as two different isoforms generated by alternative splicing (Fernandes-Alnemri et al., *Cancer Res.*, **1995**, *55*, 6045-6052).

Disclosed in the PCT publication are the polypeptide and nucleotide sequences of caspase 7 as are recombinant molecules encoding caspase 7, host cells transformed with said recombinant molecules and antibodies against the caspase 7 protein (Su and Lippke, **1997**).

Caspase 7 has been localized to human chromosome 10q25, a region which includes tumor suppressor genes believed to be associated with tumor progression in malignant gliomas and melanomas (Bullrich et al., *Genomics*, **1996**, *36*, 362-365). In addition, increased activation of caspase 7 with concomitant induction of apoptosis has been noted in several cancers including prostate cancer and leukemias (King et al., *Leukemia*, **1998**, *12*, 1553-1560; Marcelli et al., *Cancer Res.*, **1998**, *58*, 76-83; Marcelli et al., *Cancer Res.*, **1999**, *59*, 382-390).

Furthermore, it has been shown that caspase 7 affects other downstream pathways via cleavage of protein substrates involved in the stress response (Germain et al., *J. Biol. Chem.*, **1999**, *274*, 28379-28384) and iflammation (Behrensdorf et al., *FEBS Lett.*, **2000**, *466*, 143-147).

Collectively, these data suggest that modulation of caspase 7 would render opportunity to treat patients with various cancers, inflammatory conditions and deregulated apoptotic pathologic conditions.

Several types of broad-spectrum caspase inhbitors have been identified for the treatment of deregulated bone

metabolism (Harada et al., **2000**; Reszka, **1999**), as immunomodulatory agents (Gunasekera et al., **2000**) and as combination therapies for the regulation of blood cholesterol (Reszka, **1999**). These non-specific caspase inhibitors fall into three main classes; peptide-based molecules that mimic caspase substrates, small molecules and naturally-occurring caspase inhibitors or decoys (Deveraux et al., *Embo J.*, **1998**, *17*, 2215-2223; Dong et al., *Biochem. J.*, **2000**, *347 Pt 3*, 669-677; Gunasekera et al., **2000**; Harada et al., **2000**; Reszka, **1999**; Reszka, **1999**; Robidoux et al., **2000**; Spruce et al., **1999**).

Strategies aimed at modulating caspase 7 function have involved the use of antibodies and molecules that block upstream entities such as the death receptors and broad-spectrum caspase inhibitors. Bowen et al. demonstrated the role of caspase 7 in apoptosis using a 20-mer phosphodiester antisense oligonucleotide targeted to caspase 7. In this study it was shown that antisense depletion of caspase 7 slowed the onset of apoptosis in TSU-Prp1 prostate cancer cells. This antisense oligonucleotide, however, did not inhibit apoptosis to any greater extent than peptide inhibitors of cysteine proteases in general (Bowen et al., *Cell Death Differ.*, **1999**, *6*, 394-401).

Currently there exists a need to identify methods of modulating apoptosis for the therapeutic treatment of human diseases and it is believed that caspases modulators will be integral to these methods.

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of gene expression and cellular processes.

There are no known therapeutic agents which effectively inhibit the synthesis of caspase 7 and the present invention provides compositions and methods for modulating caspase 7 expression, including modulation of aberrant forms of caspase 7, including alternatively spliced forms.

SUMMARY OF THE INVENTION

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding caspase 7, and which modulate the expression of caspase 7. Pharmaceutical and other compositions comprising the compounds of the invention are also provided. Further provided are methods of modulating the expression of caspase 7 in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of caspase 7 by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in modulating the function of nucleic acid molecules encoding caspase 7, ultimately modulating the amount of caspase 7 produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding caspase 7. As used herein, the terms "target nucleic acid" and "nucleic acid encoding caspase 7" encompass DNA encoding caspase 7, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA

to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of caspase 7. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding caspase 7. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in

vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding caspase 7, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding

nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the

formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the

invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to

oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this

specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl

internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and

5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular -CH_2-NH-O-CH_2-, -CH_2-N(CH_3)-O-CH_2- [known as a methylene (methylimino) or MMI backbone], -CH_2-O-N(CH_3)-CH_2-, -CH_2-N(CH_3)-N(CH_3)-CH_2- and -O-N(CH_3)-CH_2-CH_2- [wherein the native phosphodiester backbone is represented as -O-P-O-CH_2-] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are O[$(CH_2)_n$O]$_m$$CH_3$, O$(CH_2)_n$$OCH_3$, O$(CH_2)_n$$NH_2$, O$(CH_2)_n$$CH_3$, O$(CH_2)_n$$ONH_2$, and O$(CH_2)_n$ON[$(CH_2)_n$$CH_3$)]$_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al.,

Helv. Chim. Acta, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_2)_2$, also described in examples hereinbelow.

A further prefered modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methelyne (-CH_2-)$_n$ group bridging the 2' oxygen atom and the 3' or 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-$CH=CH_2$), 2'-O-allyl (2'-O-CH_2-$CH=CH_2$) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural"

nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and*

Applications, pages 289-302, Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of

oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J.*

Pharmacol. Exp. Ther., **1996**, *277*, 923-937. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly

oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for

such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules. The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active

form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66*, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and

phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the

like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of caspase 7 is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding caspase 7, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding caspase 7 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of caspase 7 in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions

of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid,

palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Prefered bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate,. Prefered fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also prefered are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly prefered combination is the

sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999) each of which is incorporated herein by reference in their entirety.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and

liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a

system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., **1988**, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these

formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient

amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, *in: Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., **1989**, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, **1985**, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol

decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385; Ho *et al.*, *J. Pharm. Sci.*, **1996**, *85*, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or

oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract, vagina, buccal cavity and other areas of administration.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the

advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), **1988**, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, *Biochem. Biophys. Res. Commun.*, ***1987,*** *147*, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, *Journal of Controlled Release*, **1992,** *19*, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (*e.g.* as a solution or as an emulsion) were ineffective (Weiner *et al.*, *Journal of Drug Targeting*, **1992**, *2*, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al.*, *Antiviral Research*, **1992**, *18*, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al. *S.T.P.Pharma. Sci.*, **1994**, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least

for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, *FEBS Letters,* ***1987,*** *223*, 42; Wu *et al.*, *Cancer Research,* **1993,** *53*, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, **1987,** *507*, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, **1988,** *85*, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al.*).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn.*, **1980,** *53*, 2778) described liposomes comprising a nonionic detergent, $2C_{12}15G$, that contains a PEG moiety. Illum *et al.* (*FEBS Lett.*, **1984**, *167*, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (*FEBS Lett.*, **1990**, *268*, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta*, **1990,** *1029*, 91) extended such observations to other PEG-

derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.* they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without

fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and

sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, **1988**, p. 285).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92). Each of the above mentioned

classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, *J. Pharm. Pharmacol.*, **1988**, *40*, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof (*e.g.*, methyl, isopropyl and *t*-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., **1992**, *44*, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, **1996**, pp. 934-935).

Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Swinyard, Chapter 39 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, **1990**, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33; Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, **1992**, *263*, 25; Yamashita *et al.*, *J. Pharm. Sci.*, **1990**, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and

homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Buur *et al.*, *J. Control Rel.*, **1990**, *14*, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used

herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, *Antisense Res. Dev.*, **1995**, *5*, 115-121; Takakura *et al.*, *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6*, 177-183).

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*,

magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc.*).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example,

antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine,

taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. **1987**, pp. 1206-1228, Berkow *et al.*, eds., Rahway, N.J. When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body

of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, **1993**, *36*, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S$_N$2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard

methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of

product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and

2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated

with NH_3 gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was

chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine

O^2-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried

(40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P_2O_5 under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH_2Cl_2 (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH_2Cl_2 and the combined organic phase was washed with water, brine and dried over anhydrous Na_2SO_4. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine

5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH_2Cl_2). Aqueous $NaHCO_3$ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na_2SO_4, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% $NaHCO_3$ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and evaporated to dryness . The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH_2Cl_2 to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

2'-O-(dimethylaminooxyethyl)-5-methyluridine

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminooxyethyl]-5-methyluridine (1.40g, 2.4mmol) and

stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH_2Cl_2). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH_2Cl_2 to get 2'-O-(dimethylaminooxyethyl)-5-methyluridine (766mg, 92.5%).

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine

2'-O-(dimethylaminooxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P_2O_5 under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH_2Cl_2 (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P_2O_5 under high vacuum overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N^1,N^1-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous $NaHCO_3$ (40mL). Ethyl acetate layer was dried over anhydrous Na_2SO_4 and

concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH_2-O-CH_2-N(CH_2)$_2$, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O^2-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy) ethyl)]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH_2Cl_2 (2x200 mL). The combined CH_2Cl_2 layers are washed with saturated $NaHCO_3$ solution, followed by saturated NaCl

solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH_2Cl_2:Et_3N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl)]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH_2Cl_2 (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated

synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-

methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate

internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid

expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 5 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

T-24 cells:

The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg,

MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

b.END cells:

The mouse brain endothelial cell line b.END was obtained from Dr. Werner Risau at the Max Plank Instititute (Bad Nauheim, Germany). b.END cells were routinely cultured in DMEM, high glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 3000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated

with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 µL OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCC**GTCATCGCT**CCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide transfection experiments.

Example 10

Analysis of oligonucleotide inhibition of caspase 7 expression

Antisense modulation of caspase 7 expression can be

assayed in a variety of ways known in the art. For example, caspase 7 mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are taught in, for example, Ausubel, F.*M. et al.*, *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., **1996**. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Protein levels of caspase 7 can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to caspase 7 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., **1997**. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., **1997**.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., **1998**. Western blot

(immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., **1997**. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., **1991**.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, **1996**, *42*, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., **1993**. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 µL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 µL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 µL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 100 µL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 µL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13

Real-time Quantitative PCR Analysis of caspase 7 mRNA Levels

Quantitation of caspase 7 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard

curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM $MgCl_2$, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLD™, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 µL total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene

whose expression is constant, or by quantifying total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, *Analytical Biochemistry*, **1998**, *265*, 368-374.

In this assay, 175 µL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human caspase 7 were designed to hybridize to a human caspase 7 sequence, using published sequence information (GenBank accession number U37448, incorporated herein as SEQ ID NO:3). For human caspase 7 the PCR primers were:
forward primer: ATTGGTGGTAACAGTGGTAGAGTCAT (SEQ ID NO: 4)
reverse primer: CCCTTGGCTGTGTTTTGTCA (SEQ ID NO: 5) and the PCR probe was: FAM-TTGCACTTGGCAAAAAGAATCCCAATG-TAMRA (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were:
forward primer: CAACGGATTTGGTCGTATTGG (SEQ ID NO: 7)
reverse primer: GGCAACAATATCCACTTTACCAGAGT (SEQ ID NO: 8) and the PCR probe was: 5' JOE-CGCCTGGTCACCAGGGCTGCT- TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Probes and primers to mouse caspase 7 were designed to hybridize to a mouse caspase 7 sequence, using published sequence information (GenBank accession number U67321, incorporated herein as SEQ ID NO:10). For mouse caspase 7

the PCR primers were:
forward primer: TGAGGAGGACCACAGCAACTC (SEQ ID NO:11)
reverse primer: TTCCCGTAAATCAGGTCCTCTTC (SEQ ID NO: 12) and
the PCR probe was: FAM-CTGCGTCCTGCTGAGCCACGG-TAMRA
(SEQ ID NO: 13) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.
For mouse GAPDH the PCR primers were:
forward primer: GGCAAATTCAACGGCACAGT (SEQ ID NO: 14)
reverse primer: GGGTCTCGCTCCTGGAAGCT (SEQ ID NO: 15) and the PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC- TAMRA 3' (SEQ ID NO: 16) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of caspase 7 mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then robed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

To detect human caspase 7, a human caspase 7 specific

probe was prepared by PCR using the forward primer ATTGGTGGTAACAGTGGTAGAGTCAT (SEQ ID NO: 4) and the reverse primer CCCTTGGCTGTGTTTTGTCA (SEQ ID NO: 5). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

To detect mouse caspase 7, a mouse caspase 7 specific probe was prepared by PCR using the forward primer TGAGGAGGACCACAGCAACTC (SEQ ID NO:11) and the reverse primer TTCCCGTAAATCAGGTCCTCTTC (SEQ ID NO: 12). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15

Antisense inhibition of human caspase 7 expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human caspase 7 RNA, using published sequences (GenBank accession number U37448, incorporated herein as SEQ ID NO: 3, GenBank accession number U67206, incorporated herein as SEQ ID NO: 17, GenBank accession number U67319, incorporated herein as SEQ ID NO: 18, and GenBank accession number U37449, incorporated herein as SEQ ID NO: 19). The oligonucleotides are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten

2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human caspase 7 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of human caspase 7 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
135138	5'UTR	3	5	cggctgggactggcacagtc	45	20
135140	Coding	3	48	aatacagccctgatcatctg	50	21
135141	Coding	3	84	atcttcatttgctgaatcct	33	22
135142	Coding	3	94	catccactgaatcttcattt	46	23
135143	Coding	3	104	tctggcttagcatccactga	90	24
135144	Coding	3	111	ggaccggtctggcttagcat	41	25
135145	Coding	3	138	cttactgaagagggacggta	53	26
135146	Coding	3	145	tcttcttcttactgaagagg	38	27
135147	Coding	3	168	gatggatcgcatggtgacat	40	28
135148	Coding	3	230	ttgcccagcttttcaaaatt	69	29
135149	Coding	3	332	cttcggaagcacttgaagag	67	30
135150	Coding	3	338	cccaggcttcggaagcactt	91	31
135151	Coding	3	344	tcaaaacccaggcttcggaa	83	32
135152	Coding	3	354	gacaatcacgtcaaaaccca	62	33
135153	Coding	3	371	caagagcagtcattatagac	69	34
135154	Coding	3	425	gcatttgtatggtcctcttc	67	35
135155	Coding	3	496	gtgtgacaccatctttccca	80	36
135156	Coding	3	567	gaagagtttgggtttctcta	44	37
135157	Coding	3	577	cctgaatgaagaagagtttg	36	38
135158	Coding	3	713	taatagcctggaaccgtgga	70	39
135159	Coding	3	716	gagtaatagcctggaaccgt	48	40
135160	Coding	3	745	accaggagcctcttcctggg	22	41
135161	Coding	3	751	gcacaaaccaggagcctctt	90	42
135162	Coding	3	778	gctcctccaggatggagcag	20	43
135163	Coding	3	792	caggtcttttccgtgctcct	80	44
135164	Coding	3	807	gatctgcatgatttccaggt	61	45
135165	Coding	3	862	ggtcatcagactgagactca	63	46
135166	Coding	3	911	gtgagcatggagaccacaca	82	47
135167	Coding	3	930	actgaagtagagttccttgg	58	48
135168	Stop	3	944	tgatatggctattgactgaa	77	49

	Codon					
135169	3'UTR	3	971	cattgcttctcagctagaat	77	50
135170	3'UTR	3	977	gtgacccattgcttctcagc	71	51
135171	3'UTR	3	1075	agtttcttccctgttgaatc	69	52
135172	3'UTR	3	1116	aaagtctctgaaaattcaga	21	53
135173	3'UTR	3	1229	cagaagtcacttctgttgca	68	54
135174	3'UTR	3	1237	gctttctccagaagtcactt	62	55
135175	3'UTR	3	1265	ccaccaattgcagtggacac	79	56
135176	3'UTR	3	1268	ttaccaccaattgcagtgga	72	57
135177	3'UTR	3	1363	acattctgcaataaagagca	73	58
135178	3'UTR	3	1370	aatacccacatttctgcaata	60	59
135179	3'UTR	3	1372	tcaatacccacattctgcaa	79	60
135180	3'UTR	3	1407	tgccctaagccaatgaaaaa	42	61
135181	3'UTR	3	1452	agctttttctcctctaactc	77	62
135182	3'UTR	3	1504	atggtttctgttttccagac	87	63
135183	3'UTR	3	1551	tgtaagtcaatttcctgtat	75	64
135184	3'UTR	3	1615	attatcctcatagccctggg	71	65
135185	3'UTR	3	1663	tcctctggatgatcccacta	90	66
135186	3'UTR	3	1721	ttccacaatccattggtggt	66	67
135187	3'UTR	3	1747	ggctttgttcttgtcatggc	56	68
135188	3'UTR	3	1781	cttatctgtctcagctcact	39	69
135189	3'UTR	3	1783	ctcttatctgtctcagctca	75	70
135190	3'UTR	3	1803	gataggtgagacaaggtatt	14	71
135191	3'UTR	3	1861	tgaggtggcttccagatagg	62	72
135192	3'UTR	3	1899	tcctgcctcactgtcccttg	65	73
135193	3'UTR	3	1939	tcacattatgggccaggctt	67	74
135194	3'UTR	3	1948	tacttatgttcacattatgg	71	75
135195	3'UTR	3	2006	ctatctgacaacccaatgaa	64	76
135196	3'UTR	3	2069	attttaaagctataacagat	25	77
135197	3'UTR	3	2077	ccagatatattttaaagcta	86	78
135198	3'UTR	3	2109	tcaaaataaggcttggaata	56	79
135199	3'UTR	3	2290	aagcaaccacatttttaatc	53	80
135200	5'UTR	17	78	cgcacgcatagtccctggag	42	81
135201	5'UTR	17	135	ctggctcgggatagtgcgct	35	82
135202	5'UTR	17	144	gcaccgcccctggctcggga	0	83
135203	5'UTR	17	220	cctttagcgaaagcgaaact	29	84
135204	5'UTR	17	319	gttcttttccaagtgccact	42	85
135205	5'UTR	17	347	aaatcccactgctaccaccg	32	86
135206	5'UTR	18	41	atgtatcccttcctccgagg	1	87
135207	5'UTR	18	123	atactttctcctccctgtcc	0	88
135208	5'UTR	18	126	cttatactttctcctccctg	0	89
135209	Coding	18	597	gcgcctcggcatctttgtct	62	90
135210	5'UTR	19	14	tgaaggaagtacccacccag	25	91
135211	Start Codon	19	130	catcccacaaggctgatccg	41	92
135212	Start Codon	19	135	tctgccatcccacaaggctg	49	93
135213	Coding	19	574	catattttcttctccatggc	85	94
135214	Coding	19	634	aggttgcagtcttccagagat	36	95
135215	Stop Codon	3	955	gaatgtacccctgatatggc	65	96

As shown in Table 1, SEQ ID NOs 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40,

42, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 72, 73, 74, 75, 76, 78, 79, 80, 81, 82, 85, 86, 90, 92, 93, 94, 95 and 96 demonstrated at least 30% inhibition of human caspase 7 expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

Example 16

Antisense inhibition of mouse caspase 7 expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap.

In accordance with the present invention, a second series of oligonucleotides were designed to target different regions of the mouse caspase 7 RNA, using published sequences (GenBank accession number U67321, incorporated herein as SEQ ID NO: 10, GenBank accession number Y13088, incorporated herein as SEQ ID NO: 97, and GenBank accession number AI116328, incorporated herein as SEQ ID NO: 98). The oligonucleotides are shown in Table 2. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on mouse caspase 7 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates

"no data".

Table 2

Inhibition of mouse caspase 7 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
135156	Coding	10	1108	gaagagtttgggtttctcta	46	37
135157	Coding	10	1118	cctgaatgaagaagagtttg	46	38
135460	5'UTR	97	40	gtgcctgcagcccgggaaag	33	99
135461	5'UTR	97	95	tgaaagcaaaagtcccggag	66	100
135462	Start Codon	97	182	atcatcggtcatcgttccca	54	101
135463	Coding	97	413	cgctttgtcgaagttcttgt	74	102
135464	Coding	97	419	acctgtcgctttgtcgaagt	76	103
135465	Coding	97	425	gtccatacctgtcgctttgt	65	104
135466	Coding	97	446	tttgtccgtcccattccgga	78	105
135467	Coding	97	474	ggaagcacttgaagagggcc	82	106
135468	Coding	97	598	ctcagcaggacgcaggcgaa	68	107
135469	Coding	97	603	cgtggctcagcaggacgcag	80	108
135470	Coding	97	665	atgagctgtcagatccttta	72	109
135471	Coding	97	672	ccctaaaatgagctgtcaga	62	110
135472	Coding	97	679	cggtctcccctaaaatgagc	80	111
135473	Coding	97	686	tttgcatcggtctcccctaa	85	112
135474	Coding	97	692	cagggttttgcatcggtctc	81	113
135475	Coding	97	772	ggccccgagtcagcctggat	62	114
135476	Coding	97	847	accgtggagtaagcaaagag	53	115
135477	Coding	97	852	ctggaaccgtggagtaagca	47	116
135478	Coding	97	902	ggcctgcacaaaccaggagc	90	117
135479	Coding	97	909	agcagagggcctgcacaaac	64	118
135480	Coding	97	917	caggatggagcagagggcct	19	119
135481	Coding	97	922	tcattcaggatggagcagag	46	120
135482	Coding	97	929	gccatgctcattcaggatgg	60	121
135483	Coding	97	937	aggtccttgccatgctcatt	64	122
135484	Coding	97	959	ggtcaggatctgcatgatct	81	123
135485	Coding	97	967	ttcaccctggtcaggatctg	62	124
135486	Coding	97	972	tgtcgttcaccctggtcagg	78	125
135487	Coding	97	982	ctggccaccctgtcgttcac	77	126
135488	Coding	97	1003	tcagactgggactcgaagtg	72	127
135489	Coding	97	1057	gtgagcatggacaccataca	62	128
135490	Coding	97	1064	ctctttggtgagcatggaca	70	129
135491	Stop Codon	97	1090	aagggtggtcaacggctgaa	82	130
135492	3'UTR	97	1169	tctggatattttgagagcat	86	131
135493	3'UTR	97	1175	aacatttctggatattttga	71	132
135494	3'UTR	97	1263	cttagaggaagagcatgaag	58	133
135495	3'UTR	97	1330	ggtcatgctcacagcatgca	72	134
135496	3'UTR	97	1446	agcagccacctttgccaatg	71	135
135497	3'UTR	97	1461	tgagaacaaccactcagcag	67	136
135498	3'UTR	97	1480	aaaatctgccctaagccact	68	137

135499	3'UTR	97	1539	ttctatatactggaatatta	73	138
135500	3'UTR	97	1599	attcccctgtattgacccttc	79	139
135501	3'UTR	97	1606	taagtgaattccctgtattg	76	140
135502	3'UTR	97	1621	tctcaattacccgagtaagt	88	141
135503	3'UTR	97	1719	tctagaaggctgccctgcca	87	142
135504	3'UTR	97	1727	ttctagtctctagaaggctg	61	143
135505	3'UTR	97	1806	tgactcagttctggcttggt	79	144
135506	3'UTR	97	1850	gcgctggtggatatgggcac	69	145
135507	3'UTR	97	1879	ttggctttcaggcaccttgg	83	146
135508	3'UTR	97	1887	ctcactagttggctttcagg	81	147
135509	3'UTR	97	1897	tccctaggccctcactagtt	53	148
135510	3'UTR	97	1917	tgtagcacagggtagcatgg	75	149
135511	3'UTR	97	2004	acgccagaggacatggttat	69	150
135512	3'UTR	97	2033	cagtgaatagtgaatgaata	54	151
135513	3'UTR	97	2071	actcttcagcacctgtgtgg	90	152
135514	3'UTR	97	2081	gaaatgggttactcttcagc	77	153
135515	3'UTR	97	2096	tcttgtatacaaagtgaaat	51	154
135516	3'UTR	97	2180	cagaggtaaccaaagattat	69	155
135517	3'UTR	97	2211	agatcttcgcctaaattgtc	79	156
135518	3'UTR	97	2304	aaataggaacatctgtcaac	65	157
135519	5'UTR	10	41	gtatgaaaattcctgtgatt	29	158
135520	5'UTR	10	86	tttccacctgccaattctac	51	159
135521	5'UTR	10	162	gtgaaagtgtgctactgttg	40	160
135522	5'UTR	10	203	tgaaaactgcagatttgatg	29	161
135523	5'UTR	10	268	atagccatgtaatataatgt	31	162
135524	5'UTR	10	311	ttcaatcactgtagtctaag	27	163
135525	5'UTR	10	324	ctgtacgaagaacttcaatc	37	164
135526	5'UTR	10	331	ttgatggctgtacgaagaac	50	165
135527	5'UTR	10	393	aaggaaacctttttcatgcct	19	166
135528	Start Codon	10	456	attcaaagcagcagagaggg	0	167
135529	Start Codon	10	471	acttgggctgtgaacattca	24	168
135530	Start Codon	10	474	aacacttgggctgtgaacat	28	169
135531	Coding	10	484	ggactctgagaacacttggg	29	170
135532	Coding	10	508	gggcagcaactcagtttttg	0	171
135533	Exon	98	239	ctgcggtccccaccagtggg	67	172
135534	Exon	98	313	gacctccacaaacctcgctt	0	173
135535	Exon	98	320	cattccggacctccacaaac	15	174

As shown in Table 2, SEQ ID NOs 37, 38, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 159, 160, 164, 165 and 172 demonstrated at least 35% inhibition of mouse caspase 7 expression in this experiment and are therefore preferred.

The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

Example 17

Western blot analysis of caspase 7 protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to caspase 7 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

What is claimed is:

1. A compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding caspase 7, wherein said compound specifically hybridizes with and inhibits the expression of caspase 7.

2. The compound of claim 1 which is an antisense oligonucleotide.

3. The compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 42, 44, 45, 46, 47, 48, 49, 50, 51, 52, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 72, 73, 74, 75, 76, 78, 79, 80, 81, 82, 85, 86, 90, 92, 93, 94, 95, 96, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 159, 160, 164, 165 or 172.

4. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

5. The compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.

6. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

7. The compound of claim 6 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

8. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

9. The compound of claim 8 wherein the modified nucleobase is a 5-methylcytosine.

10. The compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

11. A compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding caspase 7.

12. A composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

14. The composition of claim 12 wherein the compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of caspase 7 in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of caspase 7 is inhibited.

16. A method of treating an animal having a disease or condition associated with caspase 7 comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of caspase 7 is inhibited.

17. The method of claim 16 wherein the disease or condition is an inflammatory condition.

18. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

19. The method of claim 18 wherein the hyperproliferative disorder is cancer.

20. The method of claim 16 wherein the disease or condition is a bone metabolism or cholesterol disorder.

ABSTRACT

Antisense compounds, compositions and methods are provided for modulating the expression of caspase 7. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding caspase 7. Methods of using these compounds for modulation of caspase 7 expression and for treatment of diseases associated with expression of caspase 7 are provided.

DOCKET NO.: RTS-0201

"Express Mail" Label No.: EL263653945US
Date of Deposit: 9/11/2000

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **Antisense Modulation of caspase 7 Expression** the specification of which:

(XX) is attached hereto.

( ) was filed on_____as Application Serial No._____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	Number	Date Filed	Priority Claimed			
			Yes		No	
			Yes		No	
			Yes		No	

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented)

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Provisional Application No.	Filing Date

DOCKET NO.: RTS-0201

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Herb Boswell, Registration No. 27,311; Laurel Spear Bernstein, Registration No. 37,280; Neil S. Bartfeld, Registration No. 39,901; and April C. Logan, Registration No. 33,950, of Isis Pharmaceuticals, Inc.; and Jane Massey Licata, Registration No. 32,257, and Kathleen A. Tyrrell, Registration No. 38,350 of the firm of Law Offices of Jane Massey Licata, 66 East Main Street, Marlton NJ 08053.

Address all telephone calls and correspondence to:

Jane Massey Licata
Law Offices of Jane Massey Licata
66 East Main Street
Marlton NJ 08053
(856) 810-1515

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1	Full Name: Hong Zhang	Inventor's Signature:	Date: 9-6-00
	Residence: 3339 Cadencia St Carlsbad CA 92009	Citizenship: USA	
	Post Office Address:same as above		
2	Full Name: Andrew T. Watt	Inventor's Signature:	Date: 09/07/00
	Residence: 1500 Shadowridge Dr.. Apt. 154 Vista CA 92083	Citizenship: USA	
	Post Office Address:same as above		

SEQUENCE LISTING

<110> Hong Zhang
Andrew T. Watt

<120> ANTISENSE MODULATION OF CASPASE 7 EXPRESSION

<130> RTS-0201

<160> 174

<210> 1
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 1
tccgtcatcg ctcctcaggg 20

<210> 2
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 2
atgcattctg cccccaagga 20

<210> 3
<211> 2309
<212> DNA
<213> Homo sapiens

<220>
<221> CDS

<222> (44)...(955)

<400> 3

```
gagagactgt gccagtccca gccgccctac cgccgtggga acg atg gca gat gat      55
                                                Met Ala Asp Asp
                                                  1

cag ggc tgt att gaa gag cag ggg gtt gag gat tca gca aat gaa gat     103
Gln Gly Cys Ile Glu Glu Gln Gly Val Glu Asp Ser Ala Asn Glu Asp
  5                  10                  15                  20

tca gtg gat gct aag cca gac cgg tcc tcg ttt gta ccg tcc ctc ttc     151
Ser Val Asp Ala Lys Pro Asp Arg Ser Ser Phe Val Pro Ser Leu Phe
                 25                  30                  35

agt aag aag aag aaa aat gtc acc atg cga tcc atc aag acc acc cgg     199
Ser Lys Lys Lys Lys Asn Val Thr Met Arg Ser Ile Lys Thr Thr Arg
             40                  45                  50

gac cga gtg cct aca tat cag tac aac atg aat ttt gaa aag ctg ggc     247
Asp Arg Val Pro Thr Tyr Gln Tyr Asn Met Asn Phe Glu Lys Leu Gly
         55                  60                  65

aaa tgc atc ata ata aac aac aag aac ttt gat aaa gtg aca ggt atg     295
Lys Cys Ile Ile Ile Asn Asn Lys Asn Phe Asp Lys Val Thr Gly Met
     70                  75                  80

ggc gtt cga aac gga aca gac aaa gat gcc gag gcg ctc ttc aag tgc     343
Gly Val Arg Asn Gly Thr Asp Lys Asp Ala Glu Ala Leu Phe Lys Cys
 85                  90                  95                 100

ttc cga agc ctg ggt ttt gac gtg att gtc tat aat gac tgc tct tgt     391
Phe Arg Ser Leu Gly Phe Asp Val Ile Val Tyr Asn Asp Cys Ser Cys
                105                 110                 115

gcc aag atg caa gat ctg ctt aaa aaa gct tct gaa gag gac cat aca     439
Ala Lys Met Gln Asp Leu Leu Lys Lys Ala Ser Glu Glu Asp His Thr
            120                 125                 130

aat gcc gcc tgc ttc gcc tgc atc ctc tta agc cat gga gaa gaa aat     487
Asn Ala Ala Cys Phe Ala Cys Ile Leu Leu Ser His Gly Glu Glu Asn
        135                 140                 145

gta att tat ggg aaa gat ggt gtc aca cca ata aag gat ttg aca gcc     535
```

```
Val Ile Tyr Gly Lys Asp Gly Val Thr Pro Ile Lys Asp Leu Thr Ala
    150                 155                 160

cac ttt agg ggg gat aga tgc aaa acc ctt tta gag aaa ccc aaa ctc      583
His Phe Arg Gly Asp Arg Cys Lys Thr Leu Leu Glu Lys Pro Lys Leu
165                 170                 175                 180

ttc ttc att cag gct tgc cga ggg acc gag ctt gat gat ggc atc cag      631
Phe Phe Ile Gln Ala Cys Arg Gly Thr Glu Leu Asp Asp Gly Ile Gln
                185                 190                 195

gcc gac tcg ggg ccc atc aat gac aca gat gct aat cct cga tac aag      679
Ala Asp Ser Gly Pro Ile Asn Asp Thr Asp Ala Asn Pro Arg Tyr Lys
            200                 205                 210

atc cca gtg gaa gct gac ttc ctc ttc gcc tat tcc acg gtt cca ggc      727
Ile Pro Val Glu Ala Asp Phe Leu Phe Ala Tyr Ser Thr Val Pro Gly
        215                 220                 225

tat tac tcg tgg agg agc cca gga aga ggc tcc tgg ttt gtg caa gcc      775
Tyr Tyr Ser Trp Arg Ser Pro Gly Arg Gly Ser Trp Phe Val Gln Ala
    230                 235                 240

ctc tgc tcc atc ctg gag gag cac gga aaa gac ctg gaa atc atg cag      823
Leu Cys Ser Ile Leu Glu Glu His Gly Lys Asp Leu Glu Ile Met Gln
245                 250                 255                 260

atc ctc acc agg gtg aat gac aga gtt gcc agg cac ttt gag tct cag      871
Ile Leu Thr Arg Val Asn Asp Arg Val Ala Arg His Phe Glu Ser Gln
                265                 270                 275

tct gat gac cca cac ttc cat gag aag aag cag atc ccc tgt gtg gtc      919
Ser Asp Asp Pro His Phe His Glu Lys Lys Gln Ile Pro Cys Val Val
            280                 285                 290

tcc atg ctc acc aag gaa ctc tac ttc agt caa tag ccatatcagg           965
Ser Met Leu Thr Lys Glu Leu Tyr Phe Ser Gln
        295                 300

ggtacattct agctgagaag caatgggtca ctcattaatg aatcacattt ttttatgctc   1025

ttgaaatatt cagaaattct ccaggatttt aatttcagga aaatgtattg attcaacagg   1085

gaagaaactt tctggtgctg tcttttgttc tctgaatttt cagagacttt tttataatgt   1145
```

tattcatttg gtgactgtgt aactttctct taagattaat tttctctttg tatgtctgtt 1205

accttgttaa tagacttaat acatgcaaca gaagtgactt ctggagaaag ctcatggctg 1265

tgtccactgc aattggtggt aacagtggta gagtcatgtt tgcacttggc aaaaagaatc 1325

ccaatgtttg acaaaacaca gccaagggga tatttactgc tctttattgc agaatgtggg 1385

tattgagtgt gatttgaatg atttttcatt ggcttagggc agattttcat gcaaaagttc 1445

tcatatgagt tagaggagaa aaagcttaat gattctgata tgtatccatc aggatccagt 1505

ctggaaaaca gaaaccattc taggtgtttc aacagaggga gtttaataca ggaaattgac 1565

ttacatagat gataaaagag aagccaaaca gcaagaagct gttaccacac ccagggctat 1625

gaggataatg ggaagaggtt tggtttcctg tgtccagtag tgggatcatc cagaggagct 1685

ggaaccatgg tgggggctgc ctagtgggag ttaggaccac caatggattg tggaaaatgg 1745

agccatgaca agaacaaagc cactgactga gatggagtga gctgagacag ataagagaat 1805

accttgtctc acctatcctg ccctcacatc ttccaccagc accttactgc ccaggcctat 1865

ctggaagcca cctcaccaag gaccttggaa gagcaaggga cagtgaggca ggagaagaac 1925

aagaaatgga tgtaagcctg gcccataatg tgaacataag taatcactaa tgctcaacaa 1985

tttatccatt caatcattta ttcattgggt tgtcagatag tctatgtatg tgtaaaacaa 2045

tctgttttgg ctttatgtgc aaaatctgtt atagctttaa aatatatctg gaacttttta 2105

gattattcca agccttattt tgagtaaata tttgttactt ttagttctat aagtgaggaa 2165

gagtttatgg caaagatttt tggcactttg ttttcaagat ggtgttatct tttgaattct 2225

tgataaatga ctgtttttttt ctgcctaata gtaactggtt aaaaaacaaa tgttcatatt 2285

tattgattaa aaatgtggtt gctt 2309

<210> 4
<211> 26

<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 4
attggtggta acagtggtag agtcat 26

<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 5
cccttggctg tgttttgtca 20

<210> 6
<211> 27
<212> DNA
<213> Artificial Sequence

<223> PCR Probe

<400> 6
ttgcacttgg caaaaagaat cccaatg 27

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 7
caacggattt ggtcgtattg g 21

<210> 8
<211> 26

<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 8
ggcaacaata tccactttac cagagt 26

<210> 9
<211> 21
<212> DNA
<213> Artificial Sequence

<223> PCR Probe

<400> 9
cgcctggtca ccagggctgc t 21

<210> 10
<211> 2006
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (474)...(1496)

<400> 10
agctcagtga ggctgatgtg tactgcacat ttaaaaaaaa aatcacagga attttcatac 60

aatgaataaa accacaacaa tacatgtaga attggcaggt ggaaaagagc cagcaagggc 120

tcaaactaat cactcacttt ccctcttcag catagttcaa ccaacagtag cacactttca 180

cctacaaatc ttaaagtagc tccatcaaat ctgcagtttt cacattattg aaaatgtctg 240

tcacataggt acaaatttag aatcatcaca ttatattaca tggctattct aggtcatcta 300

tagatcagat cttagactac agtgattgaa gttcttcgta cagccatcaa aaagggacac 360

atgatcatta cctactgtta gctcacatct aaaggcatga aaaggtttcc ttttttttcaa 420

```
ctgacccaaa cactttaccc caatagtgcc aggttccctc tctgctgctt tga atg      476
                                                          Met
                                                            1

ttc aca gcc caa gtg ttc tca gag tcc ttt aca aaa act gag ttg ctg      524
Phe Thr Ala Gln Val Phe Ser Glu Ser Phe Thr Lys Thr Glu Leu Leu
              5                  10                  15

ccc tcg acc ctt gcg gag gac gga cgc tgc cgt ggg ctc ctg gcc gcc      572
Pro Ser Thr Leu Ala Glu Asp Gly Arg Cys Arg Gly Leu Leu Ala Ala
         20                  25                  30

gcc gtg gga acg atg acc gat gat cag gac tgt gct gcg gag ctg gaa      620
Ala Val Gly Thr Met Thr Asp Asp Gln Asp Cys Ala Ala Glu Leu Glu
     35                  40                  45

aag gtg gat tct tcc agc gaa gac gga gtt gac gcc aag cca gac cgc      668
Lys Val Asp Ser Ser Ser Glu Asp Gly Val Asp Ala Lys Pro Asp Arg
 50                  55                  60                  65

tcc tct atc atc tcc tct att ctc ttg aag aag aag aga aat gcc tct      716
Ser Ser Ile Ile Ser Ser Ile Leu Leu Lys Lys Lys Arg Asn Ala Ser
                 70                  75                  80

gcg ggc ccc gtc agg acc ggc cgg gac cga gtg ccc act tat ctg tac      764
Ala Gly Pro Val Arg Thr Gly Arg Asp Arg Val Pro Thr Tyr Leu Tyr
             85                  90                  95

cgc atg gat ttc cag aag atg ggt aaa tgc atc atc ata aac aac aag      812
Arg Met Asp Phe Gln Lys Met Gly Lys Cys Ile Ile Ile Asn Asn Lys
        100                 105                 110

aac ttc gac aaa gcg aca ggt atg gac gtc cgg aat ggg acg gac aaa      860
Asn Phe Asp Lys Ala Thr Gly Met Asp Val Arg Asn Gly Thr Asp Lys
    115                 120                 125

gat gca ggg gcc ctc ttc aag tgc ttc caa aac ctg ggt ttt gaa gta      908
Asp Ala Gly Ala Leu Phe Lys Cys Phe Gln Asn Leu Gly Phe Glu Val
130                 135                 140                 145

acc gtc cac aat gac tgc tct tgt gca aag atg caa gat ctg ctt aga      956
Thr Val His Asn Asp Cys Ser Cys Ala Lys Met Gln Asp Leu Leu Arg
                150                 155                 160
```

```
aaa gcc tct gag gag gac cac agc aac tcg gcc tgc ttc gcc tgc gtc    1004
Lys Ala Ser Glu Glu Asp His Ser Asn Ser Ala Cys Phe Ala Cys Val
            165                 170                 175

ctg ctg agc cac ggg gaa gag gac ctg att tac ggg aaa gat ggc gtg    1052
Leu Leu Ser His Gly Glu Glu Asp Leu Ile Tyr Gly Lys Asp Gly Val
        180                 185                 190

aca ccc ata aag gat ctg aca gct cat ttt agg gga gac cga tgc aaa    1100
Thr Pro Ile Lys Asp Leu Thr Ala His Phe Arg Gly Asp Arg Cys Lys
    195                 200                 205

acc ctg tta gag aaa ccc aaa ctc ttc ttc att cag gca tgc cga ggg    1148
Thr Leu Leu Glu Lys Pro Lys Leu Phe Phe Ile Gln Ala Cys Arg Gly
210                 215                 220                 225

acg gag ctc gac gat gga atc cag gct gac tcg ggg ccc atc aac gac    1196
Thr Glu Leu Asp Asp Gly Ile Gln Ala Asp Ser Gly Pro Ile Asn Asp
                230                 235                 240

att gac gct aat ccc cgc aac aag atc ccg gtg gaa gcc gac ttc ctc    1244
Ile Asp Ala Asn Pro Arg Asn Lys Ile Pro Val Glu Ala Asp Phe Leu
            245                 250                 255

ttt gct tac tcc acg gtt cca ggt tat tac tca tgg agg aac cca ggg    1292
Phe Ala Tyr Ser Thr Val Pro Gly Tyr Tyr Ser Trp Arg Asn Pro Gly
        260                 265                 270

aaa ggc tcc tgg ttt gtg cag gcc ctc tgc tcc atc ctg aat gag cat    1340
Lys Gly Ser Trp Phe Val Gln Ala Leu Cys Ser Ile Leu Asn Glu His
    275                 280                 285

ggc aag gac ctc gag atc atg cag atc ctg acc agg gtg aac gac agg    1388
Gly Lys Asp Leu Glu Ile Met Gln Ile Leu Thr Arg Val Asn Asp Arg
290                 295                 300                 305

gtg gcc agg cac ttc gag tcc cag tct gat gat cca cgc ttc aac gag    1436
Val Ala Arg His Phe Glu Ser Gln Ser Asp Asp Pro Arg Phe Asn Glu
                310                 315                 320

aag aag cag atc ccg tgt atg gtg tcc atg ctc acc aaa gag ctg tac    1484
Lys Lys Gln Ile Pro Cys Met Val Ser Met Leu Thr Lys Glu Leu Tyr
            325                 330                 335
```

ttc agc cgt tga ccacccttca gctgagaacc tgccgccgtt cgttgatgaa 1536
Phe Ser Arg
340

tccagttttt attttatttt tgttccgatg ctctcaaaat atccagaaat gttgagggga 1596

tttaatttca ggaaagtcta gatttttttt ttttgtttaa taactttgtt catctgatga 1656

cttcatgctc ttcctctaag gttgatttcc tgtttctgtt tctttttttc tttgtcgtct 1716

cgctgagtgc atgctgtgag catgacctct ggagaagaca ttggcaatga cgtctcagtt 1776

gaacttggca aagagaatcc cagctcttga tgaaagaata cagctgcgac acctgttggc 1836

ctccattggc aaaggtggct gctgagtggt tgttctcagt ggcttagggc agatttttaa 1896

gccgaccttc ccaggtggct gagagaagac gacagttaat attccagtat atagaaccca 1956

atccagaaaa taagccatcc taggaatatc ggtgcagaag ggtcaataca 2006

<210> 11
<211> 21
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 11
tgaggaggac cacagcaact c 21

<210> 12
<211> 23
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 12
ttcccgtaaa tcaggtcctc ttc 23

<210> 13

<211> 21
<212> DNA
<213> Artificial Sequence

<223> PCR Probe

<400> 13
ctgcgtcctg ctgagccacg g 21

<210> 14
<211> 20
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 14
ggcaaattca acggcacagt 20

<210> 15
<211> 20
<212> DNA
<213> Artificial Sequence

<223> PCR Primer

<400> 15
gggtctcgct cctggaagct 20

<210> 16
<211> 27
<212> DNA
<213> Artificial Sequence

<223> PCR Probe

<400> 16
aaggccgaga atgggaagct tgtcatc 27

<210> 17

<211> 2661
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (387)...(1298)

<400> 17
cgcccgcgcg cgggctcaac tttgtagagc gaggggccaa cttggcagag cgcgcggcca 60

gctttgcaga gagcgccctc cagggactat gcgtgcgggg acacgggtcg ctttgggctc 120

ttccacccct gcggagcgca ctatcccgag ccaggggcgg tgcaagcccc gcccggccct 180

acccagggcg gctcctccct ccgcagcgcc gagactttta gtttcgcttt cgctaaaggg 240

gccccagacc cttgctgcgg agcgacggag agagactgtg ccagtcccag ccgccctacc 300

gccgtgggaa cggcaggaag tggcacttgg aaaagaacac cagctgcggt ggtagcagtg 360

ggatttgtgc ttcttatgtt acccag atg gca gat gat cag ggc tgt att gaa 413
Met Ala Asp Asp Gln Gly Cys Ile Glu
1 5

gag cag ggg gtt gag gat tca gca aat gaa gat tca gtg gat gct aag 461
Glu Gln Gly Val Glu Asp Ser Ala Asn Glu Asp Ser Val Asp Ala Lys
10 15 20 25

cca gac cgg tcc tcg ttt gta ccg tcc ctc ttc agt aag aag aag aaa 509
Pro Asp Arg Ser Ser Phe Val Pro Ser Leu Phe Ser Lys Lys Lys Lys
30 35 40

aat gtc acc atg cga tcc atc aag acc acc cgg gac cga gtg cct aca 557
Asn Val Thr Met Arg Ser Ile Lys Thr Thr Arg Asp Arg Val Pro Thr
45 50 55

tat cag tac aac atg aat ttt gaa aag ctg ggc aaa tgc atc ata ata 605
Tyr Gln Tyr Asn Met Asn Phe Glu Lys Leu Gly Lys Cys Ile Ile Ile
60 65 70

aac aac aag aac ttt gat aaa gtg aca ggt atg ggc gtt cga aac gga 653
Asn Asn Lys Asn Phe Asp Lys Val Thr Gly Met Gly Val Arg Asn Gly
75 80 85

```
aca gac aaa gat gcc gag gcg ctc ttc aag tgc ttc cga agc ctg ggt      701
Thr Asp Lys Asp Ala Glu Ala Leu Phe Lys Cys Phe Arg Ser Leu Gly
 90                  95                  100                 105

ttt gac gtg att gtc tat aat gac tgc tct tgt gcc aag atg caa gat      749
Phe Asp Val Ile Val Tyr Asn Asp Cys Ser Cys Ala Lys Met Gln Asp
                110                 115                 120

ctg ctt aaa aaa gct tct gaa gag gac cat aca aat gcc gcc tgc ttc      797
Leu Leu Lys Lys Ala Ser Glu Glu Asp His Thr Asn Ala Ala Cys Phe
            125                 130                 135

gcc tgc atc ctc tta agc cat gga gaa gaa aat gta att tat ggg aaa      845
Ala Cys Ile Leu Leu Ser His Gly Glu Glu Asn Val Ile Tyr Gly Lys
        140                 145                 150

gat ggt gtc aca cca ata aag gat ttg aca gcc cac ttt agg ggg gat      893
Asp Gly Val Thr Pro Ile Lys Asp Leu Thr Ala His Phe Arg Gly Asp
    155                 160                 165

aga tgc aaa acc ctt tta gag aaa ccc aaa ctc ttc ttc att cag gct      941
Arg Cys Lys Thr Leu Leu Glu Lys Pro Lys Leu Phe Phe Ile Gln Ala
170                 175                 180                 185

tgc cga ggg acc gag ctt gat gat ggc atc cag gcc gac tcg ggg ccc      989
Cys Arg Gly Thr Glu Leu Asp Asp Gly Ile Gln Ala Asp Ser Gly Pro
                190                 195                 200

atc aat gac aca gat gct aat cct cga tac aag atc cca gtg gaa gct     1037
Ile Asn Asp Thr Asp Ala Asn Pro Arg Tyr Lys Ile Pro Val Glu Ala
            205                 210                 215

gac ttc ctc ttc gcc tat tcc acg gtt cca ggc tat tac tca tgg agg     1085
Asp Phe Leu Phe Ala Tyr Ser Thr Val Pro Gly Tyr Tyr Ser Trp Arg
        220                 225                 230

agc cca gga aga ggc tcc tgg ttt gtg caa gcc ctc tgc tcc atc ctg     1133
Ser Pro Gly Arg Gly Ser Trp Phe Val Gln Ala Leu Cys Ser Ile Leu
    235                 240                 245

gag gag cac gga aaa gac ctg gaa atc atg cag atc ctc acc agg gtg     1181
Glu Glu His Gly Lys Asp Leu Glu Ile Met Gln Ile Leu Thr Arg Val
250                 255                 260                 265
```

```
aat gac aga gtt gcc agg cac ttt gag tct cag tct gat gac cca cac    1229
Asn Asp Arg Val Ala Arg His Phe Glu Ser Gln Ser Asp Asp Pro His
                270                 275                 280

ttc cat gag aag aag cag atc ccc tgt gtg gtc tcc atg ctc acc aag    1277
Phe His Glu Lys Lys Gln Ile Pro Cys Val Val Ser Met Leu Thr Lys
            285                 290                 295

gaa ctc tac ttc agt caa tag ccatatcagg ggtacattct agctgagaag      1328
Glu Leu Tyr Phe Ser Gln
        300

caatgggtca ctcattaatg aatcacattt ttttatgctc ttgaaatatt cagaaattct  1388

ccaggatttt aatttcagga aaatgtattg attcaacagg gaagaaactt tctgggtgct  1448

gtcttttgtt ctctgaattt tcagagactt tttttataat gttattcatt tggtgactgt  1508

gkaactttct cttaagatta attttctctt tgtatgtctg ttaccttgtt aatagactta  1568

atacatgcaa cagaagtgac ttctggagaa agctcatggc tgtgtccact gcaattggtg  1628

gcaacagtgg cagagtcatg tttgcacttg gcaaaaagaa tcccaatgtt tgacaaacac  1688

agccaagggg atatttactg ctctttattg cagaatgtgg gtattgagtg tgatttgaat  1748

gatttttcat tggcttaggg cagattttca tgcaaaagtt ctcatatgag ttagaggaga  1808

aaaagcttaa tgattctgat atgtatccat caggatccag tctggaaaac agaaaccatt  1868

ctaggtgttt caacagaggg agtttaatac aggaaattga cttacataga tgatataaga  1928

gaacccaaac agcaagaagc tgttaccaca cccaggtcta tgaggataat gggaagaggt  1988

ttggtttcct gtgtccagta gtgggatcat ccagaggagc tggaaccatg gtgggggctg  2048

cctagtggga gttaggacca ccaatggatt gtggaaaatg gagccatgac aagaacaaaa  2108

ccactgactg agatggagtg agctgagaca gataagagaa taccttggtc tcacctatcc  2168

tgccctcaca tcttccacca gcaccttact gcccaggcct atctggaagc cacctcacca  2228

aggaccttgg aagagcaagg gacagtgagg caggagaaga acaagaaatg gatgtaagcc  2288
```

tggcccataa tgtgaacata agtaatcact aatgctcaac aatttatcca ttcaatcatt 2348

tattcattgg gttgtcagat agtctatgta tgtgtaaaac aatctgtttt ggctttatgt 2408

ccaaaatctg ttatagcttt aaaatatatc tggaactttt tagattattc caagccttat 2468

tttgagtaaa tatttgttac ttttagttct ataagtgagg aagagtttat ggcaaagatt 2528

tttggcactt tgtttttcaag atggtgttat cttttgaatt cttgataaat gactgttttt 2588

ttctgcctaa tagtaactgg ttaaaaaaca aatgttcata tttattgatt aaaaatgtgg 2648

ttgcttaatt cct 2661

<210> 18
<211> 2602
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (229)...(1239)

<400> 18
gtggggtaaa acagcagaga tcaatgagat cagagcacac cctcggagga agggatacat 60

gacaaatgcc tgaacggaga gagggagtga actgtgcaaa cacacagcca ggagttttcc 120

aaggacaggg aggagaaagt ataaggcctg ctgtaccctc gatgcaaaac atgagaaagc 180

cgactgtgcc agtcccagcc gccctaccgc cgtgggaacg atgctgta atg gac tgt 237
Met Asp Cys
1

gtt ggt tgg cct cca ggc agg aag tgg cac ttg gaa aag aac acc agc 285
Val Gly Trp Pro Pro Gly Arg Lys Trp His Leu Glu Lys Asn Thr Ser
5 10 15

tgc ggt ggt agc agt ggg att tgt gct tct tat gtt acc cag atg gca 333
Cys Gly Gly Ser Ser Gly Ile Cys Ala Ser Tyr Val Thr Gln Met Ala
20 25 30 35

```
gat gat cag ggc tgt att gaa gag cag ggg gtt gag gat tca gca aat     381
Asp Asp Gln Gly Cys Ile Glu Glu Gln Gly Val Glu Asp Ser Ala Asn
                 40                  45                  50

gaa gat tca gtg gat gct aag cca gac cgg tcc tcg ttt gta ccg tcc     429
Glu Asp Ser Val Asp Ala Lys Pro Asp Arg Ser Ser Phe Val Pro Ser
             55                  60                  65

ctc ttc agt aag aag aag aaa aat gtc acc atg cga tcc atc aag acc     477
Leu Phe Ser Lys Lys Lys Lys Asn Val Thr Met Arg Ser Ile Lys Thr
         70                  75                  80

acc cgg gac cga gtg cct aca tat cag tac aac atg aat ttt gaa aag     525
Thr Arg Asp Arg Val Pro Thr Tyr Gln Tyr Asn Met Asn Phe Glu Lys
     85                  90                  95

ctg ggc aaa tgc atc ata ata aac aac aag aac ttt gat aaa gtg aca     573
Leu Gly Lys Cys Ile Ile Ile Asn Asn Lys Asn Phe Asp Lys Val Thr
100                 105                 110                 115

ggt atg ggc gtt cga aac gga aca gac aaa gat gcc gag gcg ctc ttc     621
Gly Met Gly Val Arg Asn Gly Thr Asp Lys Asp Ala Glu Ala Leu Phe
                120                 125                 130

aag tgc ttc cga agc ctg ggt ttt gac gtg att gtc tat aat gac tgc     669
Lys Cys Phe Arg Ser Leu Gly Phe Asp Val Ile Val Tyr Asn Asp Cys
            135                 140                 145

tct tgt gcc aag atg caa gat ctg ctt aaa aaa gct tct gaa gag gac     717
Ser Cys Ala Lys Met Gln Asp Leu Leu Lys Lys Ala Ser Glu Glu Asp
        150                 155                 160

cat aca aat gcc gcc tgc ttc gcc tgc atc ctc tta agc cat gga gaa     765
His Thr Asn Ala Ala Cys Phe Ala Cys Ile Leu Leu Ser His Gly Glu
    165                 170                 175

gaa aat gta att tat ggg aaa gat ggt gtc aca cca ata aag gat ttg     813
Glu Asn Val Ile Tyr Gly Lys Asp Gly Val Thr Pro Ile Lys Asp Leu
180                 185                 190                 195

aca gcc cac ttt agg ggg gat aga tgc aaa acc ctt tta gag aaa ccc     861
Thr Ala His Phe Arg Gly Asp Arg Cys Lys Thr Leu Leu Glu Lys Pro
                200                 205                 210
```

aaa ctc ttc ttc att cag gct tgc cga ggg acc gag ctt gat gat ggc 909
Lys Leu Phe Phe Ile Gln Ala Cys Arg Gly Thr Glu Leu Asp Asp Gly
215 220 225

atc cag gcc gac tcg ggg ccc atc aat gac aca gat gct aat cct cga 957
Ile Gln Ala Asp Ser Gly Pro Ile Asn Asp Thr Asp Ala Asn Pro Arg
230 235 240

tac aag atc cca gtg gaa gct gac ttc ctc ttc gcc tat tcc acg gtt 1005
Tyr Lys Ile Pro Val Glu Ala Asp Phe Leu Phe Ala Tyr Ser Thr Val
245 250 255

cca ggc tat tac tca tgg agg agc cca gga aga ggc tcc tgg ttt gtg 1053
Pro Gly Tyr Tyr Ser Trp Arg Ser Pro Gly Arg Gly Ser Trp Phe Val
260 265 270 275

caa gcc ctc tgc tcc atc ctg gag gag cac gga aaa gac ctg gaa atc 1101
Gln Ala Leu Cys Ser Ile Leu Glu Glu His Gly Lys Asp Leu Glu Ile
280 285 290

atg cag atc ctc acc agg gtg aat gac aga gtt gcc agg cac ttt gag 1149
Met Gln Ile Leu Thr Arg Val Asn Asp Arg Val Ala Arg His Phe Glu
295 300 305

tct cag tct gat gac cca cac ttc cat gag aag aag cag atc ccc tgt 1197
Ser Gln Ser Asp Asp Pro His Phe His Glu Lys Lys Gln Ile Pro Cys
310 315 320

gtg gtc tcc atg ctc acc aag gaa ctc tac ttc agt caa tag ccatatcagg 1249
Val Val Ser Met Leu Thr Lys Glu Leu Tyr Phe Ser Gln
325 330 335

ggtacattct agctgagaag caatgggtca ctcattaatg aatcacattt ttttatgctc 1309

ttgaaatatt cagaaattct ccaggatttt aatttcagga aaatgtattg attcaacagg 1369

gaagaaactt tctgggtgct gtcttttgtt ctctgaattt tcagagactt tttttataat 1429

gttattcatt tggtgactgt gkaactttct cttaagatta attttctctt tgtatgtctg 1489

ttaccttgtt aatagactta atacatgcaa cagaagtgac ttctggagaa agctcatggc 1549

tgtgtccact gcaattggtg gcaacagtgg cagagtcatg tttgcacttg gcaaaaagaa 1609

tcccaatgtt tgacaaacac agccaagggg atatttactg ctctttattg cagaatgtgg 1669

gtattgagtg tgatttgaat gatttttcat tggcttaggg cagattttca tgcaaaagtt 1729

ctcatatgag ttagaggaga aaaagcttaa tgattctgat atgtatccat caggatccag 1789

tctggaaaac agaaaccatt ctaggtgttt caacagaggg agtttaatac aggaaattga 1849

cttacataga tgatataaga gaacccaaac agcaagaagc tgttaccaca cccaggtcta 1909

tgaggataat gggaagaggt ttggtttcct gtgtccagta gtgggatcat ccagaggagc 1969

tggaaccatg gtgggggctg cctagtggga gttaggacca ccaatggatt gtggaaaatg 2029

gagccatgac aagaacaaaa ccactgactg agatggagtg agctgagaca gataagagaa 2089

taccttggtc tcacctatcc tgccctcaca tcttccacca gcaccttact gcccaggcct 2149

atctggaagc cacctcacca aggaccttgg aagagcaagg gacagtgagg caggagaaga 2209

acaagaaatg gatgtaagcc tggcccataa tgtgaacata agtaatcact aatgctcaac 2269

aatttatcca ttcaatcatt tattcattgg gttgtcagat agtctatgta tgtgtaaaac 2329

aatctgtttt ggctttatgt ccaaaatctg ttatagcttt aaaatatatc tggaacttttt 2389

tagattattc caagccttat tttgagtaaa tatttgttac ttttagttct ataagtgagg 2449

aagagtttat ggcaaagatt tttggcactt tgttttcaag atggtgttat cttttgaatt 2509

cttgataaat gactgttttt ttctgcctaa tagtaactgg ttaaaaaaca aatgttcata 2569

tttattgatt aaaaatgtgg ttgcttaatt cct 2602

<210> 19
<211> 2378
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (147)...(908)

<400> 19

```
gcaagctggg ctgctgggtg ggtacttcct tcaaagctga gggagcgtcc tacgcccacg    60

cgcgcaggag ggcgcccccc gcaaagcaac gtctaggaga ccacagtgga tgccacagcg   120

ggccccgaagc ggatcagcct tgtgggg atg gca gat gat cag ggc tgt att gaa   173
                               Met Ala Asp Asp Gln Gly Cys Ile Glu
                                 1               5

gag cag ggg gtt gag gat tca gca aat gaa gat tca gtg gat gct aag     221
Glu Gln Gly Val Glu Asp Ser Ala Asn Glu Asp Ser Val Asp Ala Lys
 10                  15                  20                  25

cca gac cgg tcc tcg ttt gta ccg tcc ctc ttc agt aag aag aag aaa     269
Pro Asp Arg Ser Ser Phe Val Pro Ser Leu Phe Ser Lys Lys Lys Lys
                 30                  35                  40

aat gtc acc atg cga tcc atc aag acc acc cgg gac cga gtg cct aca     317
Asn Val Thr Met Arg Ser Ile Lys Thr Thr Arg Asp Arg Val Pro Thr
             45                  50                  55

tat cag tac aac atg aat ttt gaa aag ctg ggc aaa tgc atc ata ata     365
Tyr Gln Tyr Asn Met Asn Phe Glu Lys Leu Gly Lys Cys Ile Ile Ile
         60                  65                  70

aac aac aag aac ttt gat aaa gtg aca ggt atg ggc gtt cga aac gga     413
Asn Asn Lys Asn Phe Asp Lys Val Thr Gly Met Gly Val Arg Asn Gly
     75                  80                  85

aca gac aaa gat gcc gag gcg ctc ttc aag tgc ttc cga agc ctg ggt     461
Thr Asp Lys Asp Ala Glu Ala Leu Phe Lys Cys Phe Arg Ser Leu Gly
 90                  95                 100                 105

ttt gac gtg att gtc tat aat gac tgc tct tgt gcc aag atg caa gat     509
Phe Asp Val Ile Val Tyr Asn Asp Cys Ser Cys Ala Lys Met Gln Asp
                110                 115                 120

ctg ctt aaa aaa gct tct gaa gag gac cat aca aat gcc gcc tgc ttc     557
Leu Leu Lys Lys Ala Ser Glu Glu Asp His Thr Asn Ala Ala Cys Phe
            125                 130                 135

gcc tgc atc ctc tta agc cat gga gaa gaa aat atg gaa tct tgc tct     605
Ala Cys Ile Leu Leu Ser His Gly Glu Glu Asn Met Glu Ser Cys Ser
        140                 145                 150
```

```
gtc acc cag gct gga gtg cag cgg cgt gat ctc gga aga ctg caa cct     653
Val Thr Gln Ala Gly Val Gln Arg Arg Asp Leu Gly Arg Leu Gln Pro
    155                 160                 165

cca cct ccc agg ctt gcc gag gga ccg agc ttg atg atg gca tcc agg     701
Pro Pro Pro Arg Leu Ala Glu Gly Pro Ser Leu Met Met Ala Ser Arg
170                 175                 180                 185

ccg act cgg ggc cca tca atg aca cag atg cta atc ctc gat aca aga     749
Pro Thr Arg Gly Pro Ser Met Thr Gln Met Leu Ile Leu Asp Thr Arg
                190                 195                 200

tcc cag tgg aag ctg act tcc tct tcg cct att cca cgg ttc cag gct     797
Ser Gln Trp Lys Leu Thr Ser Ser Ser Pro Ile Pro Arg Phe Gln Ala
            205                 210                 215

att act cgt gga gga gcc cag gaa gag gct cct ggt ttg tgc aag ccc     845
Ile Thr Arg Gly Gly Ala Gln Glu Glu Ala Pro Gly Leu Cys Lys Pro
        220                 225                 230

tct gct cca tcc tgg agg agc acg gaa aag acc tgg aaa tca tgc aga     893
Ser Ala Pro Ser Trp Arg Ser Thr Glu Lys Thr Trp Lys Ser Cys Arg
    235                 240                 245

tcc tca cca ggg tga atgacagagt tgccaggcac tttgagtctc agtctgatga     948
Ser Ser Pro Gly
250

cccacacttc catgagaaga agcagatccc ctgtgtggtc tccatgctca ccaaggaact  1008

ctacttcagt caatagccat atcaggggta cattctagct gagaagcaat gggtcactca  1068

ttaatgaatc acattttttt atgctcttga aatattcaga aattctccag gattttaatt  1128

tcaggaaaat gtattgattc aacagggaag aaactttctg gtgctgtctt ttgttctctg  1188

aattttcaga gacttttta taatgttatt catttggtga ctgtgtaact ttctcttaag  1248

attaattttc tctttgtatg tctgttacct tgttaataga cttaatacat gcaacagaag  1308

tgacttctgg agaaagctca tggctgtgtc cactgcaatt ggtggtaaca gtggtagagt  1368

catgtttgca cttggcaaaa agaatcccaa tgtttgacaa aacacagcca aggggatatt  1428
```

tactgctctt tattgcagaa tgtgggtatt gagtgtgatt tgaatgattt ttcattggct 1488

tagggcagat tttcatgcaa aagttctcat atgagttaga ggagaaaaag cttaatgatt 1548

ctgatatgta tccatcagga tccagtctgg aaaacagaaa ccattctagg tgtttcaaca 1608

gagggagttt aatacaggaa attgacttac atagatgata aaagagaagc caaacagcaa 1668

gaagctgtta ccacacccag ggctatgagg ataatgggaa gaggtttggt ttcctgtgtc 1728

cagtagtggg atcatccaga ggagctggaa ccatggtggg ggctgcctag tgggagttag 1788

gaccaccaat ggattgtgga aaatggagcc atgacaagaa caaagccact gactgagatg 1848

gagtgagctg agacagataa gagaatacct tgtctcacct atcctgccct cacatcttcc 1908

accagcacct tactgcccag gcctatctgg aagccacctc accaaggacc ttggaagagc 1968

aagggacagt gaggcaggag aagaacaaga aatggatgta agcctggccc ataatgtgaa 2028

cataagtaat cactaatgct caacaattta tccattcaat catttattca ttgggttgtc 2088

agatagtcta tgtatgtgta aaacaatctg ttttggcttt atgtgcaaaa tctgttatag 2148

ctttaaaata tatctggaac tttttagatt attccaagcc ttattttgag taaatatttg 2208

ttacttttag ttctataagt gaggaagagt ttatggcaaa gatttttggc actttgtttt 2268

caagatggtg ttatcttttg aattcttgat aaatgactgt ttttttctgc ctaatagtaa 2328

ctggttaaaa aacaaatgtt catatttatt gattaaaaat gtggttgctt 2378

<210> 20
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 20
cggctgggac tggcacagtc 20

<210> 21
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 21
aatacagccc tgatcatctg 20

<210> 22
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 22
atcttcattt gctgaatcct 20

<210> 23
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 23
catccactga atcttcattt 20

<210> 24
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 24
tctggcttag catccactga 20

<210> 25
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 25
ggaccggtct ggcttagcat 20

<210> 26
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 26
cttactgaag agggacggta 20

<210> 27
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 27
tcttcttctt actgaagagg 20

<210> 28
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 28
gatggatcgc atggtgacat 20

<210> 29
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 29
ttgcccagct tttcaaaatt 20

<210> 30
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 30
cttcggaagc acttgaagag 20

<210> 31
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 31
cccaggcttc ggaagcactt 20

<210> 32
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 32
tcaaaaccca ggcttcggaa 20

<210> 33
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 33
gacaatcacg tcaaaaccca 20

<210> 34
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 34
caagagcagt cattatagac 20

<210> 35
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 35
gcatttgtat ggtcctcttc 20

<210> 36
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 36
gtgtgacacc atctttccca 20

<210> 37
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 37
gaagagtttg ggtttctcta 20

<210> 38
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 38
cctgaatgaa gaagagtttg 20

<210> 39
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 39
taatagcctg gaaccgtgga 20

<210> 40
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 40
gagtaatagc ctggaaccgt 20

<210> 41
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 41
accaggagcc tcttcctggg 20

<210> 42
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 42
gcacaaacca ggagcctctt 20

<210> 43
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 43
gctcctccag gatggagcag 20

<210> 44
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 44
caggtctttt ccgtgctcct 20

<210> 45
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 45
gatctgcatg atttccaggt 20

<210> 46
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 46
ggtcatcaga ctgagactca 20

<210> 47
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 47
gtgagcatgg agaccacaca 20

<210> 48
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 48
actgaagtag agttccttgg 20

<210> 49
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 49
tgatatggct attgactgaa 20

<210> 50
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 50
cattgcttct cagctagaat 20

<210> 51
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 51
gtgacccatt gcttctcagc 20

<210> 52
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 52
agtttcttcc ctgttgaatc 20

<210> 53
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 53
aaagtctctg aaaattcaga 20

<210> 54
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 54
cagaagtcac ttctgttgca 20

<210> 55
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 55
gctttctcca gaagtcactt 20

<210> 56
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 56
ccaccaattg cagtggacac 20

<210> 57
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 57
ttaccaccaa ttgcagtgga 20

<210> 58
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 58
acattctgca ataaagagca 20

<210> 59
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 59
aatacccaca ttctgcaata 20

<210> 60
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 60
tcaataccca cattctgcaa 20

<210> 61
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 61
tgccctaagc caatgaaaaa 20

<210> 62
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 62
agctttttct cctctaactc 20

<210> 63
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 63
atggtttctg ttttccagac 20

<210> 64
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 64
tgtaagtcaa tttcctgtat 20

<210> 65
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 65
attatcctca tagccctggg 20

<210> 66
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 66
tcctctggat gatcccacta 20

<210> 67
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 67
ttccacaatc cattggtggt 20

<210> 68
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 68
ggctttgttc ttgtcatggc 20

<210> 69
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 69
cttatctgtc tcagctcact 20

<210> 70
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 70
ctcttatctg tctcagctca 20

<210> 71
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 71
gataggtgag acaaggtatt 20

<210> 72
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 72
tgaggtggct tccagatagg 20

<210> 73
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 73
tcctgcctca ctgtcccttg 20

<210> 74
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 74
tcacattatg ggccaggctt 20

<210> 75
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 75
tacttatgtt cacattatgg 20

<210> 76
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 76
ctatctgaca acccaatgaa 20

<210> 77
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 77
attttaaagc tataacagat 20

<210> 78
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 78
ccagatatat tttaaagcta 20

<210> 79
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 79
tcaaaataag gcttggaata 20

<210> 80
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 80
aagcaaccac atttttaatc 20

<210> 81
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 81
cgcacgcata gtccctggag 20

<210> 82
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 82
ctggctcggg atagtgcgct 20

<210> 83
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 83
gcaccgcccc tggctcggga 20

<210> 84
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 84
cctttagcga aagcgaaact 20

<210> 85
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 85
gttcttttcc aagtgccact 20

<210> 86
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 86
aaatcccact gctaccaccg 20

<210> 87
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 87
atgtatccct tcctccgagg 20

<210> 88
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 88
atactttctc ctccctgtcc 20

<210> 89
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 89
cttatacttt ctcctccctg 20

<210> 90
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 90
gcgcctcggc atctttgtct 20

<210> 91
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 91
tgaaggaagt acccacccag 20

<210> 92
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 92
catcccacaa ggctgatccg 20

<210> 93
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 93
tctgccatcc cacaaggctg 20

<210> 94
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 94
catattttct tctccatggc 20

<210> 95
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 95
aggttgcagt cttccgagat 20

<210> 96
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 96
gaatgtaccc ctgatatggc 20

<210> 97
<211> 2372
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (190)...(1101)

<400> 97
gcggcgggct ccgtagcgcg cgttcccgct cgcccccagc tttcccgggc tgcaggcaca 60

gccccgtccg gcccgcccgc tcctcccccc ccgcctccgg gacttttgct ttcagttttc 120

ccaaagctgc cctcgaccct tgcggaggac ggacgctgcc gtgggctcct ggccgccgcc 180

gtgggaacg atg acc gat gat cag gac tgt gct gcg gag ctg gaa aag gtg 231
Met Thr Asp Asp Gln Asp Cys Ala Ala Glu Leu Glu Lys Val
1 5 10

gat tct tcc agc gaa gac gga gtt gac gcc aag cca gac cgc tcc tct 279
Asp Ser Ser Ser Glu Asp Gly Val Asp Ala Lys Pro Asp Arg Ser Ser
15 20 25 30

atc atc tcc tct att ctc ttg aag aag aag aga aat gcc tct gcg ggc 327
Ile Ile Ser Ser Ile Leu Leu Lys Lys Lys Arg Asn Ala Ser Ala Gly
35 40 45

ccc gtc agg acc ggc cgg gac cga gtg ccc act tat ctg tac cgc atg 375
Pro Val Arg Thr Gly Arg Asp Arg Val Pro Thr Tyr Leu Tyr Arg Met
50 55 60

gat ttc cag aag atg ggt aaa tgc atc atc ata aac aac aag aac ttc 423
Asp Phe Gln Lys Met Gly Lys Cys Ile Ile Ile Asn Asn Lys Asn Phe
65 70 75

gac aaa gcg aca ggt atg gac gtc cgg aat ggg acg gac aaa gat gca 471
Asp Lys Ala Thr Gly Met Asp Val Arg Asn Gly Thr Asp Lys Asp Ala
80 85 90

ggg gcc ctc ttc aag tgc ttc caa aac ctg ggt ttt gaa gta acc gtc 519
Gly Ala Leu Phe Lys Cys Phe Gln Asn Leu Gly Phe Glu Val Thr Val
95 100 105 110

```
cac aat gac tgc tct tgt gca aag atg caa gat ctg ctt aga aaa gcc     567
His Asn Asp Cys Ser Cys Ala Lys Met Gln Asp Leu Leu Arg Lys Ala
                115                 120                 125

tct gag gag gac cac agc aac tcg gcc tgc ttc gcc tgc gtc ctg ctg     615
Ser Glu Glu Asp His Ser Asn Ser Ala Cys Phe Ala Cys Val Leu Leu
            130                 135                 140

agc cac ggg gaa gag gac ctg att tac ggg aaa gat ggc gtg aca ccc     663
Ser His Gly Glu Glu Asp Leu Ile Tyr Gly Lys Asp Gly Val Thr Pro
        145                 150                 155

ata aag gat ctg aca gct cat ttt agg gga gac cga tgc aaa acc ctg     711
Ile Lys Asp Leu Thr Ala His Phe Arg Gly Asp Arg Cys Lys Thr Leu
    160                 165                 170

tta gag aaa ccc aaa ctc ttc ttc att cag gca tgc cga ggg acg gag     759
Leu Glu Lys Pro Lys Leu Phe Phe Ile Gln Ala Cys Arg Gly Thr Glu
175                 180                 185                 190

ctc gat gat gga atc cag gct gac tcg ggg ccc atc aac gac att gac     807
Leu Asp Asp Gly Ile Gln Ala Asp Ser Gly Pro Ile Asn Asp Ile Asp
                195                 200                 205

gct aat ccc cgc aac aag atc ccg gtg gaa gcc gac ttc ctc ttt gct     855
Ala Asn Pro Arg Asn Lys Ile Pro Val Glu Ala Asp Phe Leu Phe Ala
            210                 215                 220

tac tcc acg gtt cca ggt tat tac tca tgg agg aac cca ggg aaa ggc     903
Tyr Ser Thr Val Pro Gly Tyr Tyr Ser Trp Arg Asn Pro Gly Lys Gly
        225                 230                 235

tcc tgg ttt gtg cag gcc ctc tgc tcc atc ctg aat gag cat ggc aag     951
Ser Trp Phe Val Gln Ala Leu Cys Ser Ile Leu Asn Glu His Gly Lys
    240                 245                 250

gac ctc gag atc atg cag atc ctg acc agg gtg aac gac agg gtg gcc     999
Asp Leu Glu Ile Met Gln Ile Leu Thr Arg Val Asn Asp Arg Val Ala
255                 260                 265                 270

agg cac ttc gag tcc cag tct gat gat cca cgc ttc aac gag aag aag    1047
Arg His Phe Glu Ser Gln Ser Asp Asp Pro Arg Phe Asn Glu Lys Lys
                275                 280                 285
```

cag atc ccg tgt atg gtg tcc atg ctc acc aaa gag ctg tac ttc agc 1095
Gln Ile Pro Cys Met Val Ser Met Leu Thr Lys Glu Leu Tyr Phe Ser
290 295 300

cgt tga ccacccttca gctgagaacc tgccgccgtt cgttgatgaa tccagttttt 1151
Arg

attttatttt tgttccgatg ctctcaaaat atccagaaat gttgagggga tttaatttca 1211

ggaaagtcta gatttttttt tttttgttta ataactttgt tcatctgatg acttcatgct 1271

cttcctctaa ggttgatttc ctgtttctgt ttctttttt ctttgtcgtc tcgctgagtg 1331

catgctgtga gcatgacctc tggagaagac attggcaatg acgtctcagt tgaacttggc 1391

aaagagaatc ccagctcttg atgaaagaat acagctgcga cacctgttgg cctccattgg 1451

caaaggtggc tgctgagtgg ttgttctcag tggcttaggg cagattttta agccgacctt 1511

cccaggtggc tgagagaaga cgacagttaa tattccagta tatagaaccc aatccagaaa 1571

ataagccatc ctaggaatat cggtgcagaa gggtcaatac agggaattca cttactcggg 1631

taattgagag acagtcaatc ccgttacctc acatgcctct gtggggctgt aagaggactt 1691

cggttccctt gtcatcagta agaactgtgg cagggcagcc ttctagagac tagaaccatg 1751

gaggactatg gtgattggag ccaggctcag gacaagccac tgcctgagat ggaaaccaag 1811

ccagaactga gtcagacacc aaaggcctcc ccaggcctgt gcccatatcc accagcgcct 1871

tataattcca aggtgcctga aagccaacta gtgagggcct agggaccatg ctaccctgtg 1931

ctacagagca gaggaaggga tagcaaagca ggggtagtgg tgggggtaaa cttggctcaa 1991

aatgtgaatt aaataaccat gtcctctggc gtgctacaat gtattcattc actattcact 2051

gatttgtcag atcatccatc cacacaggtg ctgaagagta acccatttca ctttgtatac 2111

aagataatgt ttttgtactt caaatacatc tggaattctt tcaaatattc caagattttt 2171

ttttctgaat aatctttggt tacctctggt tctacaaatg acaatttagg cgaagatctt 2231

agcagtttct ttccaaggta tcattttctt tggaattcgt tatacattcc tgtttttccc 2291

acctaataaa tggttgacag atgttcctat ttattgatta aaatgtgttt cagaaaaaaa 2351

aaaaaaaaaa aaaaaaaaaa a 2372

<210> 98
<211> 416
<212> DNA
<213> Mus musculus

<400> 98
aaaaaaaagc tgccctcgac ccttgcggag gacggacgct gccgtgggct cctggccgcc 60

gccgtgggaa cgatgaccga tgatcaggac tgtgctgcgg agctggaaaa ggtggattct 120

tccagcgaag acggagttga cgccaagcca gaccgctcct ctatcatctc ctctattctc 180

ttgaagaaga agagaaatgc ctctgggggc cccgtcagga ccggccggtg cgcgggtgcc 240

cactggtggg gaccgcaggg aggtggcaag tgagggagag gtgcatgggt ggagagaaga 300

ggggcgtcgg caaagcgagg tttgtggagg tccggaatgg gacggagaaa gatgcagggg 360

cgcgcttcaa gtgctgccaa aacctgggtt ttgaagtaac cgtccacaat gactgc 416

<210> 99
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 99
gtgcctgcag cccgggaaag 20

<210> 100
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 100
tgaaagcaaa agtcccggag 20

<210> 101
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 101
atcatcggtc atcgttccca 20

<210> 102
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 102
cgctttgtcg aagttcttgt 20

<210> 103
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 103
acctgtcgct ttgtcgaagt 20

<210> 104
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 104
gtccatacct gtcgctttgt 20

<210> 105
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 105
tttgtccgtc ccattccgga 20

<210> 106
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 106
ggaagcactt gaagagggcc 20

<210> 107
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 107
ctcagcagga cgcaggcgaa 20

<210> 108
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 108
cgtggctcag caggacgcag 20

<210> 109
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 109
atgagctgtc agatccttta 20

<210> 110
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 110
ccctaaaatg agctgtcaga 20

<210> 111
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 111
cggtctcccc taaaatgagc 20

<210> 112
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 112
tttgcatcgg tctcccctaa 20

<210> 113
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 113
cagggttttg catcggtctc 20

<210> 114
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 114
ggccccgagt cagcctggat 20

<210> 115
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 115
accgtggagt aagcaaagag 20

<210> 116
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 116
ctggaaccgt ggagtaagca 20

<210> 117
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 117
ggcctgcaca aaccaggagc 20

<210> 118
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 118
agcagagggc ctgcacaaac 20

<210> 119
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 119
caggatggag cagagggcct 20

<210> 120
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 120
tcattcagga tggagcagag 20

<210> 121
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 121
gccatgctca ttcaggatgg 20

<210> 122
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 122
aggtccttgc catgctcatt 20

<210> 123
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 123
ggtcaggatc tgcatgatct 20

<210> 124
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 124
ttcaccctgg tcaggatctg 20

<210> 125
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 125
tgtcgttcac cctggtcagg 20

<210> 126
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 126
ctggccaccc tgtcgttcac 20

<210> 127
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 127
tcagactggg actcgaagtg 20

<210> 128
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 128
gtgagcatgg acaccataca 20

<210> 129
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 129
ctctttggtg agcatggaca 20

<210> 130
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 130
aagggtggtc aacggctgaa 20

<210> 131
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 131
tctggatatt ttgagagcat 20

<210> 132
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 132
aacatttctg gatattttga 20

<210> 133
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 133
cttagaggaa gagcatgaag 20

<210> 134
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 134
ggtcatgctc acagcatgca 20

<210> 135
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 135
agcagccacc tttgccaatg 20

<210> 136
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 136
tgagaacaac cactcagcag 20

<210> 137
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 137
aaaatctgcc ctaagccact 20

<210> 138
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 138
ttctatatac tggaatatta 20

<210> 139
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 139
attccctgta ttgacccttc 20

<210> 140
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 140
taagtgaatt ccctgtattg 20

<210> 141
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 141
tctcaattac ccgagtaagt 20

<210> 142
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 142
tctagaaggc tgccctgcca 20

<210> 143
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 143
ttctagtctc tagaaggctg 20

<210> 144
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 144
tgactcagtt ctggcttggt 20

<210> 145
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 145
gcgctggtgg atatgggcac 20

<210> 146
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 146
ttggctttca ggcaccttgg 20

<210> 147
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 147
ctcactagtt ggctttcagg 20

<210> 148
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 148
tccctaggcc ctcactagtt 20

<210> 149
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 149
tgtagcacag ggtagcatgg 20

<210> 150
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 150
acgccagagg acatggttat 20

<210> 151
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 151
cagtgaatag tgaatgaata 20

<210> 152
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 152
actcttcagc acctgtgtgg 20

<210> 153
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 153
gaaatgggtt actcttcagc 20

<210> 154
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 154
tcttgtatac aaagtgaaat 20

<210> 155
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 155
cagaggtaac caaagattat 20

<210> 156
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 156
agatcttcgc ctaaattgtc 20

<210> 157
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 157
aaataggaac atctgtcaac 20

<210> 158
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 158
gtatgaaaat tcctgtgatt 20

<210> 159
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 159
tttccacctg ccaattctac 20

<210> 160
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 160
gtgaaagtgt gctactgttg 20

<210> 161
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 161
tgaaaactgc agatttgatg 20

<210> 162
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 162
atagccatgt aatataatgt 20

<210> 163
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 163
ttcaatcact gtagtctaag 20

<210> 164
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 164
ctgtacgaag aacttcaatc 20

<210> 165
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 165
ttgatggctg tacgaagaac 20

<210> 166
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 166
aaggaaacct tttcatgcct 20

<210> 167
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 167
attcaaagca gcagagaggg 20

<210> 168
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 168
acttgggctg tgaacattca 20

<210> 169
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 169
aacacttggg ctgtgaacat 20

<210> 170
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 170
ggactctgag aacacttggg 20

<210> 171
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 171
gggcagcaac tcagtttttg 20

<210> 172
<211> 20
<212> DNA

<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 172
ctgcggtccc caccagtggg 20

<210> 173
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 173
gacctccaca aacctcgctt 20

<210> 174
<211> 20
<212> DNA
<213> Artificial Sequence

<223> Antisense Oligonucleotide

<400> 174
cattccggac ctccacaaac 2